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Interactions between *Bacillus thuringiensis* subsp. *israelensis* and Fathead Minnows, *Pimephales promelas* Rafinesque, under Laboratory Conditions

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Interactions between *Bacillus thuringiensis* subsp. *israelensis* and fathead minnows, *Pimephales promelas*, were studied in laboratory exposures to two commercial formulations, Vectobac-G and Mosquito Attack. Mortality among fatheads exposed to 2.0×10^6 to 6.5×10^6 CFU/ml with both formulations was attributed to severe dissolved oxygen depletion due to formulation ingredients rather than to direct toxicity from the parasporal crystal. No adverse effects were observed at 6.4×10^5 CFU/ml and below. Fathead minnows rapidly accumulated high numbers of spores with 1 h of exposure to 2.2×10^5 CFU of Mosquito Attack per ml, producing whole-body counts of 4.0×10^6 CFU per fish. Comparison of counts on gastrointestinal tract samples and whole-body samples and high numbers of spores in feces indicated that ingestion was the major route of exposure. *B. thuringiensis* subsp. *israelensis* spore counts decreased rapidly after transfer of fish to clean water, with a drop of over 3 orders of magnitude in 1 day. Spores were rarely detected in fish after 8 days but were detectable in feces for over 2 weeks. These findings suggest that fish could influence the dissemination of *B. thuringiensis* subsp. *israelensis*, and possibly other microbial agents, in the aquatic environment.

Concerns over the environmental hazards of chemical pesticides have stimulated interest in development and use of microbial pest control agents (MPCAs). Because of their host specificity, MPCAs are generally regarded as potentially less harmful and thus are seen as attractive alternatives to some chemical applications within an integrated control program (5, 16). However, MPCAs, as living organisms, pose concerns related to their survival, multiplication, and dissemination in the environment which are not shared by chemical pesticides.

The discovery of *Bacillus thuringiensis* subsp. *israelensis*, which possesses specific toxicity to larval mosquitoes and black flies, quickly led to its commercial development and registration for use in aquatic environments (4, 13). It has been shown to be fairly host specific, being highly toxic to many species of mosquitoes and to black flies, slightly toxic to a few closely related species, and largely innocuous to a wide range of aquatic organisms, including fish (4, 7, 17, 18, 24). Many studies have focused on physical and environmental factors that affect its host species efficacy, including particulate levels, settling rates, flow rates, and dosing regimens (8, 10, 19).

Few studies, however, have investigated the role of nontarget biota on the fate and distribution of the agent. Brazner and Anderson (2) demonstrated that short laboratory exposures resulted in ingestion and adsorption of large numbers of spores by the amphipod *Gammarus lacustris* and in long-term retention of low levels of spores. Their study suggested that nontarget invertebrates may have significant influence on the persistence and transport of *B. thuringiensis* subsp. *israelensis* within an aquatic ecosystem.

Numerous studies have shown that the microflora of fish directly reflect the microbiological condition of their environment and are influenced by the bacterial content of both the water and food consumed (6, 11, 12, 14, 22, 23). On the basis of these reports, it is probable that fish influence the fate of an MPCA in an aquatic environment.

The present study investigated interactions between fish and *B. thuringiensis* subsp. *israelensis* as a model for spore-forming MPCAs. Laboratory exposures were conducted to measure the response of fish to different densities of two commercial *B. thuringiensis* subsp. *israelensis* formulations, the effects of exposure duration on spore uptake, and the relative contribution of surface adsorption and ingestion to the whole body levels. In addition, the retention time in fish under different feeding regimens was examined.

MATERIALS AND METHODS

Test organisms. Fathead minnows, *Pimephales promelas* Rafinesque, were obtained from the Environmental Research Laboratory-Duluth culture unit. Fish were held in 24°C Lake Superior water and fed live brine shrimp (*Artemia* sp.) nauplii twice daily.

***B. thuringiensis* subsp. *israelensis* sources, solution preparation, and enumeration of bacteria.** Two commercial *B. thuringiensis* subsp. *israelensis* spore-crystal formulations, Vectobac-G (Abbott Laboratories, Chicago, Ill.), a preparation on corncob grits, and Mosquito Attack (Reuter Laboratories, Inc., Gainesville, Va.), a wettable powder, were used. Vectobac-G stock suspensions were prepared by stirring the grits for 5 min in sterile lake water and filtering through a 0.5-mm-mesh stainless steel screen to remove the large grits. Mosquito Attack stock suspensions were prepared by stirring preweighed formulation directly into sterile lake water.

Stocks were prepared and appropriately diluted in lake water immediately before use. Water samples were generally pasteurized (65°C for 20 min) to reduce interference by indigenous microflora. *B. thuringiensis* subsp. *israelensis* density was estimated by a standard pour plate method on tryptic soy agar (TSA). Colonies showing typical colony appearance were counted after 24 to 48 h of incubation at 30°C.

Sample homogenization increased counts by an average of

three times for Vectobac-G and nine times for Mosquito Attack. Since homogenization was not routinely performed, exposure densities reported herein likely underestimate actual spores per milliliter. Nevertheless, standard plate counts produced consistent results on a formulation weight-per-volume basis. Plate counts yielded estimates of 2×10^8 to 4×10^8 CFU/g for Vectobac-G and 1×10^9 to 2×10^9 CFU/g for Mosquito Attack.

General experimental procedures. Exposure protocol generally followed that recommended for static toxicity tests (1). Spore densities tested ranged from approximately 2×10^4 to 6.5×10^6 CFU/ml (based on standard pour plates) and were estimated to be approximately 100 to 500 times the recommended application rates for both formulations. The whole-body (WB) *B. thuringiensis* subsp. *israelensis* spore content of fathead minnows, inclusive of both adsorbed and ingested spores, was measured as an indicator of *B. thuringiensis* subsp. *israelensis*-fish interaction. Survival, behavior, and growth of test animals, when appropriate, were also monitored.

At the start of each experiment, fathead minnows were randomly assigned to beakers or aquaria containing 24°C Lake Superior water. Fish were not fed for 24 h before or during exposure to bacteria. Exposure was begun by dropwise addition of stock suspension to the water surface and gentle stirring. A mid-depth water sample was removed by sterile pipette, and bacteria were counted as described above.

Quantification of bacteria in fish. After exposure for 1 to 48 h, fish were transferred to a clean chamber and rinsed in flowing lake water. Single fish, a composite of all fish from a single chamber, or a dissected tissue constituted one sample. Each sample was homogenized in 2 to 10 ml of sterile lake water with a Tissumizer (Tekmar Corp., Cincinnati, Ohio). Tissue homogenates were generally pasteurized (65°C for 20 min) to reduce background vegetative cells. Appropriate dilutions were poured into plates of TSA, and typical *B. thuringiensis* subsp. *israelensis* colonies were counted. Spore-crystal stains (20, 21) and bioassays with larval mosquitoes, *Aedes atropalpus*, were used as confirmation on randomly selected colonies. Unexposed (control) fathead minnows of the same lot were similarly processed and analyzed for *B. thuringiensis* subsp. *israelensis*. Colonies resembling *B. thuringiensis* subsp. *israelensis* were rare in control fish. Plate count results are reported on a per-fish or per-tissue basis.

Fecal samples were also analyzed with standard TSA pour plates after homogenization and, generally, pasteurization.

Exposure density experiments. The relationship between exposure density and fathead minnow survival and spore accumulation was investigated in a series of exposures with both formulations at densities from 2.0×10^4 to 6.5×10^6 CFU/ml. Survival and behavior of minnows were monitored for up to 96 h. Dissolved oxygen concentrations in the exposure solutions were measured with a dissolved oxygen meter (Yellow Springs Instrument Co., Yellow Springs, Ohio). Spore counts were made on fish exposed at densities that caused no adverse effects on survival.

Exposure time versus uptake. Five 3-week-old fathead minnows (mean weight, 0.020 g) were added to each of 16 1,000-ml beakers containing 750 ml of water. Fourteen beakers were treated with Mosquito Attack at 2.2×10^5 CFU/ml; two beakers were kept as untreated controls. After 1, 3, 6, 12, 24, and 48 h, the fish from two or four (6 h) treated beakers were sampled for quantification of *B. thuringiensis* subsp. *israelensis*. Control fish were sampled after 48 h. All fish from each beaker were counted as one sample.

Adsorption and ingestion experiments. Experiments were conducted to estimate the relative contribution of surface-adsorbed and ingested spores to WB spore counts. Quantification of adsorbed spores by common skin swab or scrape methods proved unreliable for these small fish; therefore, gills were sampled as an alternative. The entire gastrointestinal (GI) tract was sampled to measure ingestion. In the first experiment, 12 3-month-old minnows (average weight, 0.2 g) were sampled after 22 h of exposure to 2.5×10^5 CFU of Vectobac-G per ml. WB spore counts were made on six fish. The external surface of each of the remaining six fish was gently wiped to dryness with alcohol, and the gills and GI tract of each were dissected under a microscope with sterile microscissors and forceps. Each tissue was individually analyzed for *B. thuringiensis* subsp. *israelensis*. Because spore counts were higher than expected in the GI tract, samples were inadequately diluted and plates were too numerous to count. The experiment was repeated with 20 additional fish; however, only WB and GI tract samples were analyzed. To determine the influence of tissue type on spore recovery, plate counts of *B. thuringiensis* subsp. *israelensis*-spiked WB, gill, and GI tract homogenates from control fish were compared with counts on similarly spiked and homogenized water samples.

Spore retention experiments. Sixty-five 3-week-old fathead minnows (average weight, 0.025 g) were exposed in each of two 12-liter glass aquaria to 2.5×10^5 CFU of Vectobac-G per ml. After 22 h of exposure, fish from one aquarium were rinsed in flowing lake water; then five fish were randomly assigned to each of 13 500-ml flow-through aquaria (duplicate set A) receiving 36 ml of lake water per min. The procedure was repeated for the second exposure aquarium, assigning the fish to 13 identical aquaria (duplicate set B).

Fish from two aquaria, one per duplicate set, were sampled immediately after exposure (t_0) and at 1, 2, 3, 4, 8, 15, and 30 days postexposure. Fish were fed live brine shrimp twice daily beginning on the day of transfer. To minimize recontamination, aquaria were siphoned thoroughly 3 h after each feeding and the uneaten food and feces were discarded. Before the first daily feeding, feces eliminated since the previous cleaning (14 to 16 h earlier) were collected into a sterile beaker and allowed to settle. Feces in a small amount of water were transferred to a sterile test tube, diluted to 20 ml with sterile lake water, and homogenized. A 5-ml sample was removed for *B. thuringiensis* subsp. *israelensis* analysis, and the remainder was used for mosquito bioassay. Because total recovery of feces was not made, counts were used only to assess the presence and relative *B. thuringiensis* subsp. *israelensis* levels in the feces.

A similar experiment was run with Mosquito Attack to determine the influence of formulation and of feeding on *B. thuringiensis* subsp. *israelensis* retention. Forty-two 3-week-old minnows (average weight, 0.031 g) were exposed per duplicate aquarium to 1.5×10^5 CFU/ml under conditions identical to those described above. After exposure, six fish per duplicate set were sampled to determine initial *B. thuringiensis* subsp. *israelensis* accumulation, and three were transferred to each flow-through aquarium. Fish in half the aquaria per duplicate set were fed throughout the post-exposure period. Fish in the other half were starved for the first 8 days postexposure. All aquaria were siphoned twice daily. At 1, 2, 3, 4, 8, and 16 days postexposure, fed and unfed fish from one aquarium per feeding regimen were sampled and analyzed for *B. thuringiensis* subsp. *israelensis*.

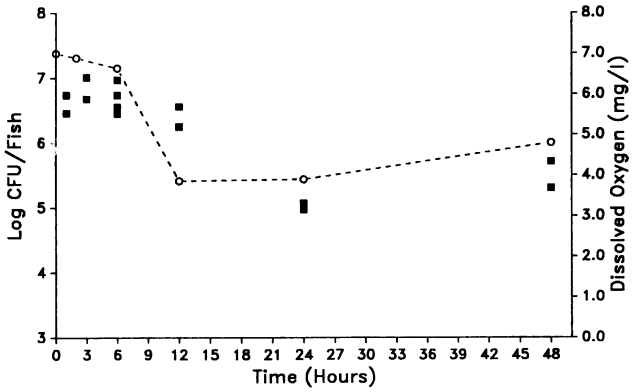

FIG. 1. WB *B. thuringiensis* subsp. *israelensis* spore content of fathead minnows (■) and dissolved oxygen concentration of exposure water (○) after various lengths of exposure to 2.2×10^5 CFU of Mosquito Attack formulation per ml. Each datum point represents the average of duplicate TSA plate counts on a five-fish composite; results are expressed on a per-fish basis.

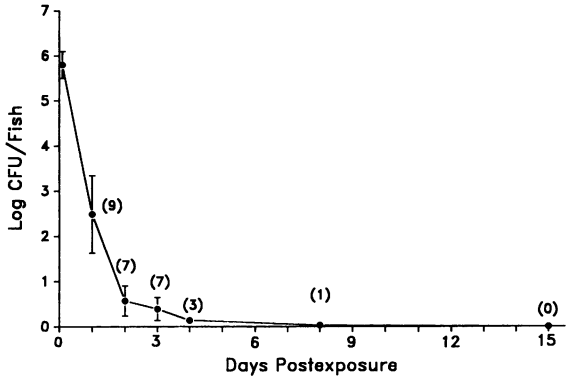

FIG. 2. WB *B. thuringiensis* subsp. *israelensis* spore content of fathead minnows after 22 h of exposure to 2.5×10^5 CFU of Vectobac-G per ml and transfer to clean water. Symbols indicate means of the log CFU per fish that were obtained from 10 fish sampled at each time. Bars indicate 95% confidence intervals (based on the *t* statistic) about means. In cases where not all samples were positive, numbers within parentheses near datum points indicate numbers of samples in which *B. thuringiensis* subsp. *israelensis* was detected.

RESULTS

Survival. Exposure to 6.5×10^6 CFU of Vectobac-G per ml resulted in complete mortality of larval fathead minnows ($n = 10$) within 24 h. Within 8 to 12 h of exposure, fish displayed signs of respiratory distress, i.e., gasping and swimming at the water surface. No mortality was seen at 6.2×10^4 and 6.4×10^5 CFU/ml in 24 h. Subsequent exposures of juvenile minnows to Vectobac-G and Mosquito Attack produced similar responses. Exposure to 2.0×10^6 CFU of Vectobac-G per ml and 6.5×10^6 CFU of Mosquito Attack per ml resulted in 60 and 100% mortality, respectively, in 96 h. No mortality or signs of stress were observed at densities of 6.0×10^5 CFU/ml and below with either formulation. Mortality corresponded to severely depleted dissolved oxygen levels at the highest densities. Dissolved oxygen measurements showed little or no apparent differences from those of controls (6.7 to 7.1 mg/liter) at 3×10^4 CFU/ml, reductions to 3.6 mg/liter at 2.5×10^5 CFU/ml, and severe depletion to 0.5 and 1.2 mg/liter at 6.5×10^6 and 2.0×10^6 CFU/ml, respectively, in 12 to 20 h for both formulations.

Density versus uptake. No significant difference in spore uptake was detected in fish exposed to 2×10^4 to 6×10^5 CFU of either formulation per ml ($P > 0.05$, *t* test). For example, 22 h of exposure to 1.2×10^4 and 2.1×10^5 CFU of Mosquito Attack per ml resulted in mean WB counts of 4.75×10^5 and 1.45×10^5 CFU per fish, respectively.

Effect of exposure time on WB spore count. Fathead minnows rapidly accumulated high levels of spores upon exposure to Mosquito Attack (Fig. 1). Fish were observed feeding on formulation particles immediately after addition. Plate counts on the two composite samples after 1 h of exposure provided a mean WB spore count of 3.99×10^6 CFU per fish. Little difference in WB spore count was observed between 1 and 12 h of exposure; the 10 samples during this interval averaged 4.4×10^6 CFU per fish. In contrast, WB estimates after 24 and 48 h were 1 to 2 orders of magnitude lower than earlier samples and averaged 1.83×10^5 CFU per fish.

Dissolved oxygen in the water declined from a mean of 7.0 mg/liter in control and predose beakers to 6.4 mg/liter in 6 h and to a low of 3.8 mg/liter in 12 h in treatment beakers. Dissolved oxygen in controls remained stable over the entire experiment.

Adsorption and ingestion experiments. Plate count results on WB, GI tract, and gill homogenates clearly demonstrated that ingestion accounted for most of the spores accumulated by fathead minnows (Table 1). The mean WB count, 1.85×10^5 CFU per fish, was 20% of that on GI tract samples, 9.71×10^5 CFU per fish; however, the means were not significantly different ($P > 0.05$, *t* test). Differences in recovery between WB and GI tract spiked homogenates (50 and 95%, respectively) and individual variability in uptake probably account for the observed difference.

The mean spore count on gills, 1.7×10^3 CFU per sample, was only 0.3% of the average WB count.

Spore retention experiments. *B. thuringiensis* subsp. *israelensis* spore counts declined exponentially after Vectobac-G-exposed minnows were transferred to clean water (Fig. 2). Within 1 day postexposure, WB counts decreased over 2,000 times from a mean of 6.3×10^5 CFU per fish to 305 CFU per fish. At 2 days, mean counts decreased to 3 CFU per fish

TABLE 1. Spore counts of WB, gill, and GI tract samples of fathead minnows exposed to Vectobac-G for 22 h

Exposure	CFU/fish or tissue, 10^3[a]		
	WB	Gill	GI tract
Trial 1 (2.5×10^5 CFU/ml)	533 (131–2,170), 6	1.71 (0.215–13.55), 6	TNTC (>650), 6
Trial 2 (1.1×10^5 CFU/ml)	185 (34–1,010), 9	NA	971 (218–4,320), 10

[a] Results are given as the mean (95% confidence interval), *n*. TNTC, Too numerous to count; NA, not analyzed. Recoveries of *B. thuringiensis* subsp. *israelensis* spores spiked into control fish tissue homogenate and compared with similarly spiked and homogenized lake water samples were as follows (mean [percentage of lake water spike], $n = 3$): lake water, 3.92×10^4 CFU/ml; WB (0.025 g of tissue per ml), 1.96×10^4 CFU/ml (50%); gill, 3.62×10^4 CFU/ml (92%); GI tract, 3.70×10^4 CFU/ml (94%).

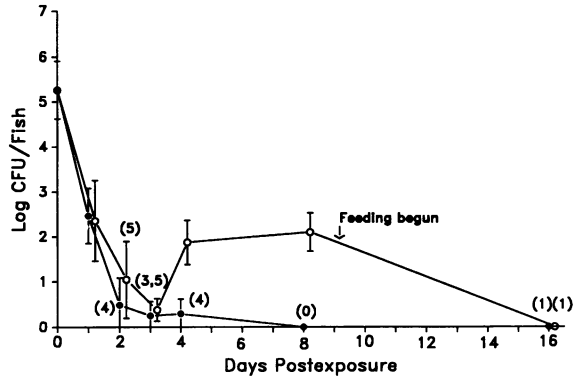

FIG. 3. WB *B. thuringiensis* subsp. *israelensis* spore content of fathead minnows after 22 h of exposure to 1.5×10^5 CFU of Mosquito Attack per ml and transfer to clean water. Symbols indicate means of the log CFU per fish that were obtained from 6 fed fish (●) and 6 unfed fish (○) at each time, except 12 fish at T_0 (all unfed). Data for unfed fish (○) are offset slightly to the right for clarity. See the legend to Fig. 2 for further details.

(range, 0 to 25 CFU per fish). By 4 days postexposure, spores were detectable in 3 of 10 fish sampled, with one to three colonies per fish. After 8 days, only one fish homogenate was positive (one colony). All samples at 15 and 30 days were negative.

In contrast to WB homogenates, fecal samples contained relatively high levels of spores throughout the first week postexposure. Samples collected during the first 3 days postexposure contained between 1×10^5 and 9×10^5 CFU per sample. Counts on feces collected 4 days postexposure averaged 5×10^4 CFU per sample, and counts on eight samples collected between 7 and 9 days ranged from 1.1×10^3 and 6.0×10^3 CFU per sample. Counts of 50 to 100 spores were estimated in 15-day fecal samples. Accurate counts on samples from 15 days and beyond were impossible because of the abundance of a non-*B. thuringiensis* subsp. *israelensis* spore former in the samples.

Mosquito larvae exposed to fecal samples collected in the first 3 days postexposure died in 12 to 18 h, showing that the *B. thuringiensis* subsp. *israelensis* crystal retained its specific toxicity during passage through the minnow GI tract. Samples produced at least partial mortality of mosquitos through day 5.

Results obtained in the Mosquito Attack retention study for fed minnows were essentially identical to those in the Vectobac-G retention study (Fig. 3). Also, spore counts from starved fish were indistinguishable from those of fed fish during the first 3 days postexposure. At 3 days postexposure, *B. thuringiensis* subsp. *israelensis* was detected in three of six fed fish and in five of six unfed fish with only one to four colonies per fish. In contrast, all six unfed fish sampled at 4 days were positive, with a mean WB count of 74 CFU per fish (95% confidence interval, 24 to 232); four fed fish yielded 1 to 6 CFU per fish. At 8 days, unfed fish had 124 CFU per fish (95% confidence interval, 46 to 333). Coprophagy by the starving fish, first noted on day 3, likely accounted for this secondary increase in *B. thuringiensis* subsp. *israelensis*. Fecal samples from the two aquaria with unfed fish sampled at 8 days had 500 and 1,500 spores. Feeding of unfed fish was begun at 9 days to prevent further starvation. One *B. thuringiensis* subsp. *israelensis* colony each was detected in one fed fish and one unfed fish at 16 days.

DISCUSSION

This study demonstrated that fathead minnows rapidly accumulated large numbers of *B. thuringiensis* subsp. *israelensis* spores, primarily through ingestion, after the addition of commercial formulations to their water. The presence of large numbers of *B. thuringiensis* subsp. *israelensis* spores in the minnow GI tract verified that the gut, which is the site of pathogenic activity in target species, had been challenged. This finding and the lack of direct adverse effect support previous findings of innocuousness of *B. thuringiensis* subsp. *israelensis* to fish species.

The observed mortality during exposures to 2.5×10^6 to 6.5×10^6 CFU/ml with both formulations is believed to be an indirect effect due to severe dissolved oxygen depletion by components of the formulations rather than specific toxicity of the parasporal crystal. The oxygen levels measured at these densities, 0.5 to 1.3 mg/liter, were lower than the 2.0-mg/liter concentration reported to cause 100% mortality of fathead minnow fry (3). Mortalities in brook trout fry exposed to another *B. thuringiensis* subsp. *israelensis* formulation, Teknar, were attributed to toxicity from xylene used in the formulation (9). Although these effects were seen only at concentrations many times the recommended application rates, these observations emphasize that potential formulation effects should be considered during environmental risk assessments for MPCA products.

Although no significant difference in spore accumulation was observed after exposure to different spore densities, WB spore numbers decreased with exposure time. WB counts showed that fish exposed for 22 h or greater had approximately 10 times fewer spores ($\sim 3.5 \times 10^5$ CFU per fish) than did those exposed for 12 h or less ($\sim 4 \times 10^6$ CFU per fish). Unpublished findings revealed that germination occurred in the fish gut and that the rate of germination observed can explain the decline in spore numbers with time.

Results of this study showing maximum clearance of *B. thuringiensis* subsp. *israelensis* spores from fathead minnows within 24 h and complete elimination in 1 to 2 weeks are consistent with those of previous laboratory studies with other fish and bacterial species. Glantz and Krantz (12) detected *Escherichia coli* in trout for 1 to 14 days after exposure to *E. coli*-dosed food and water. Similarly, Geldreich and Clarke (11) demonstrated that fecal coliforms and fecal streptococci added to tank water were retained in bluegill and carp intestinal tracts for 9 to 14 days. In a study of fecal excretion rates of food-incorporated bacteria, Lesel and Le Gac (15) found little difference in elimination of three species of enterobacteria and spores of *B. stearothermophilus* used as a transit indicator in rainbow trout. Excretion began within 1 h after feeding and several hours before solid food elimination. Excretion reached a maximum within 8 to 20 h and had generally ended in 4 to 7 days. Lesel and Le Gac suggested that the bacterial cells were associated with a liquid phase that preceded the solid food phase. This independence of transit may explain why no difference in elimination was observed between fed and unfed fathead minnows in the present study.

Results in each of these studies, including the present study, revealed that colonization of the gut did not occur and that fish acted simply as passive carriers of the bacteria.

The demonstration of significant ingestion and low-level persistence of *B. thuringiensis* subsp. *israelensis* for up to 2 weeks in the fathead minnow and the survival of *B. thuringiensis* subsp. *israelensis* in fish feces has important ecological ramifications. These findings imply that fish influence the

survival, persistence, and dissemination of *B. thuringiensis* subsp. *israelensis*, and probably other MPCA, in an aquatic ecosystem.

I suggest that laboratory exposures can be useful tools for understanding the role of fish in the fate of a bacterial agent before its release into the environment. Laboratory-derived information on uptake, retention times, and the ability of the microbe to survive and possibly multiply in the gut or feces could be incorporated into a risk assessment process and be used to signal the need for further, more in-depth studies before actual release into the environment.

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